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14. ABSTRACT
We have met all of the milestones outlined in this grant, except demonstration that the electrospun conduit supports cell growth. Composite gels are a more promising method for the brain patch therapy due to greater degree of control over shape of the three-dimensional structure, therefore the majority of our efforts have been focused on gel development. In addition to the work outlined in the original proposal, we have also determined a method for assessing diffusion of growth factors out of our gel matrices. Characterization of the release kinetics of neural-specific growth factors from gel matrices will help to inform the design of an appropriate growth-factor laden matrix for the cell delivery system. We have demonstrated expression of

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mesenchymal stem cells, collagen-chitosan matrices, neural cell differentiation

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| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | Elizabeth Orwin |
| U | U | U | SAR | | 19b. TELEPHONE NUMBER 909-621-8019 |

## Report Title

Cell Delivery System for Traumatic Brain Injury

## ABSTRACT

We have met all of the milestones outlined in this grant, except demonstration that the electrospun conduit supports cell growth. Composite gels are a more promising method for the brain patch therapy due to greater degree of control over shape of the three-dimensional structure, therefore the majority of our efforts have been focused on gel development. In addition to the work outlined in the original proposal, we have also determined a method for assessing diffusion of growth factors out of our gel matrices. Characterization of the release kinetics of neural-specific growth factors from gel matrices will help to inform the design of an appropriate growth-factor laden matrix for the cell delivery system. We have demonstrated expression of early and late neuronal protein markers in MSCs in both monolayer and 3D collagen scaffolds via both retinoic acid treatment and co-culture with astrocytes. The most promising results have been in MSCs cultured in 3D collagen scaffolds at high cell seeding densities. Co-culture with astrocytes is the most promising differentiation method due to long-term cytotoxicity with the RA method and poor neural protein expression with growth factor method.

## List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

### (a) Papers published in peer-reviewed journals (N/A for none)

**Number of Papers published in peer-reviewed journals:** 0.00

### (b) Papers published in non-peer-reviewed journals or in conference proceedings (N/A for none)

**Number of Papers published in non peer-reviewed journals:** 0.00

### (c) Presentations

Nina Bordeaux*, Kacy McKibben*, Abbygail Palmer*, Nancy Lape and Elizabeth Orwin. "Measuring the Diffusion Kinetics of Proteins through Novel Matrices." 6th Annual California Tissue Engineering Meeting at UCLA, Nov 30-Dec 1, 2007.

Madineh Sarvestani*, Jeff Manassero*, Justin Kim*, Maureen St Georges*, Nicole Esclamado* and Elizabeth Orwin. "Development of a Cell Delivery System for Traumatic Brain Injury Using Novel Matrices and Human Bone Marrow Stem Cells." 4th Annual Los Angeles Tissue Engineering Meeting, UCLA Dec. 2006.

**Number of Presentations:** 2.00

### Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

**Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):** 0

### Peer-Reviewed Conference Proceeding publications (other than abstracts):

**Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):** 0

### (d) Manuscripts

**Number of Manuscripts:** 0.00

**Number of Inventions:**

## Graduate Students

| NAME | PERCENT_SUPPORTED |
|---|---|
| **FTE Equivalent:** | |
| **Total Number:** | |

## Names of Post Doctorates

| NAME | PERCENT_SUPPORTED |
|---|---|
| **FTE Equivalent:** | |
| **Total Number:** | |

## Names of Faculty Supported

| NAME | PERCENT_SUPPORTED | National Academy Member |
|---|---|---|
| Elizabeth Orwin | 0.00 | No |
| **FTE Equivalent:** | **0.00** | |
| **Total Number:** | **1** | |

## Names of Under Graduate students supported

| NAME | PERCENT_SUPPORTED |
|---|---|
| Tanya Lewis | 0.25 |
| Cassandra Cortez | 0.25 |
| Justin Kim | 0.25 |
| Jeff Manassero | 0.25 |
| Nicole Esclamado | 0.25 |
| Arjun Kalyanpur | 0.25 |
| Clark Zhang | 0.25 |
| Maureen St. Georges-Chaumet | 0.25 |
| **FTE Equivalent:** | **2.00** |
| **Total Number:** | **8** |

## Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

| | |
|---|---|
| The number of undergraduates funded by this agreement who graduated during this period: ..... | 6.00 |
| The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields: ..... | 6.00 |
| The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields: ..... | 3.00 |
| Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale): ..... | 2.00 |
| Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering: ..... | 0.00 |
| The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense ..... | 0.00 |
| The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: ..... | 1.00 |

## Names of Personnel receiving masters degrees

NAME

**Total Number:**

## Names of personnel receiving PHDs

NAME

**Total Number:**

## Names of other research staff

NAME PERCENT_SUPPORTED

**FTE Equivalent:**

**Total Number:**

## Sub Contractors (DD882)

## Inventions (DD882)

HARVEY MUDD COLLEGE

# Cell Delivery System for Traumatic Brain Injury (SF298)

**Elizabeth Orwin, Ph.D., Isabella Wulur, M.S., Madineh Sarvestani, B.S., Nicole Esclamado, B.S.**

**Feb. 29, 2008**

## *I. Final Report Summary*

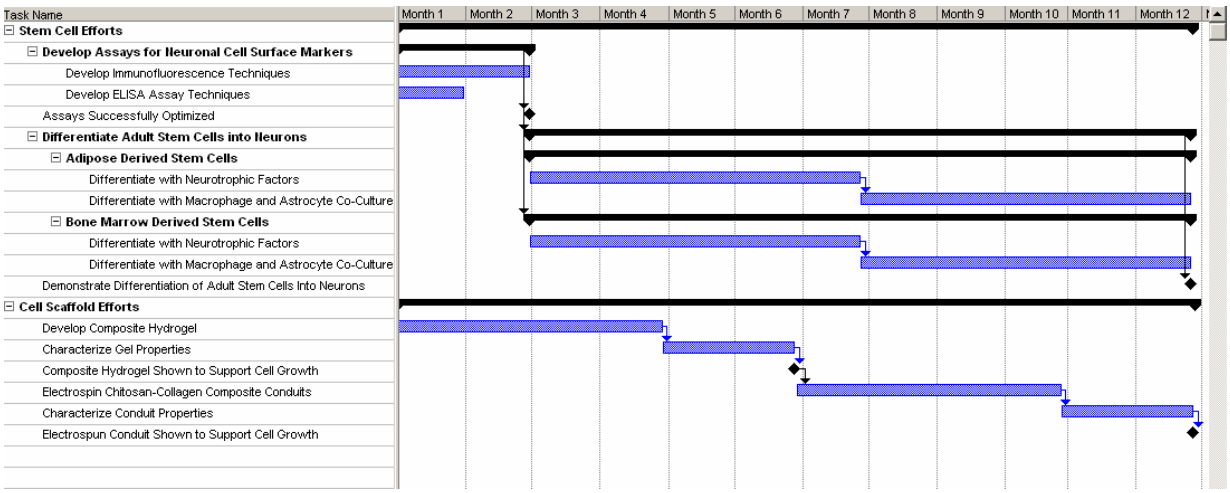
This report documents the achievements on #BAA06-9 F121 Cell Delivery System for the Treatment of Traumatic Brain Injury dated Sept 2006-Sept 2007 with a 5 month extension to Feb 2008. Specific accomplishments met under this grant include:

- Demonstrated neural protein expression in MSCs differentiated via two methods:
  - Retinoic acid –supplemented media
  - Co-culture with astrocytes
- Demonstrated increased neural protein expression in MSCs grown in 3D collagen matrices and at higher cell seeding densities
- Developed functionalized chitosan scaffolds and chitosan-collagen composite scaffolds and demonstrated cell attachment to both
- Modified existing CyQuant cell viability assay for chitosan-based materials
- Optimized immunofluorescence and Western Blot techniques for neural markers

We have met all of the milestones outlined in this grant, except demonstration that the electrospun conduit supports cell growth. Composite gels are a more promising method for the brain patch therapy due to greater degree of control over shape of the three-dimensional structure, therefore the majority of our efforts have been focused on gel development. In addition to the work outlined in the original proposal, we have also determined a method for assessing diffusion of growth factors out of our gel matrices. Characterization of the release kinetics of neural-specific growth factors from gel matrices will help to inform the design of an appropriate growth-factor laden matrix for the cell delivery system. We have demonstrated expression of early and late neuronal protein markers in MSCs in both monolayer and 3D collagen scaffolds via both retinoic acid treatment and co-culture with astrocytes. The most promising results have been in MSCs cultured in 3D collagen scaffolds at high cell seeding densities. Co-culture with astrocytes is the most promising differentiation method due to long-term cytotoxicity with the RA method and poor neural protein expression with growth factor method.

## ***II. Productivity Report***

The milestones outlined in the original proposal and the Gantt chart are listed below:




*Milestones and Summary:*

1. Month 2: Assays successfully completed

- Immunofluorescence and Western blot techniques have been optimized and verified using rat neurons as a positive control for all neural intracellular markers of interest (GFAP, MAP2, β-tubulin II and synaptophysin)
- The use of ELISA assays was determined to be not as useful in identifying neuronal markers.

2. Month 6: Demonstrate composite hydrogel supports cell growth

- Our composite chitosan-collagen hydrogel supports MSC attachment as determined by SEM and confocal microscopy of tissue samples.
- We have modified the CyQUANT cell viability assay with a proteinase K digestion step and demonstrated use of this assay on chitosan-based scaffolds.
- We have developed a method for assessing growth factor diffusion out of developed hydrogels.

3. Month 12: Demonstrate electrospun conduit supports cell growth

- We developed chitosan/collagen composite electrospun mats.
- Electrospinning of chitosan fibers proved difficult and took longer than expected. In addition, we found it easier to control the overall structure (shape, size, etc.) of the gel matrices and concentrated most of our efforts on gel development. Therefore, we did not evaluate cell viability on electrospun mats.

4. Month 12: Demonstrate differentiation of adult stem cells into neurons

- We have demonstrated expression of early and late neuronal protein markers in MSCs in both monolayer and 3D collagen scaffolds via both retinoic acid treatment and co-culture with astrocytes. The most promising results have been in MSCs cultured in 3D collagen scaffolds at high cell seeding densities.
- Most of the work in this grant was done on MSCs and not on adipose derived stem cells (ADSCs). A literature review and consultation with senior scientists in the field have indicated that ADSCs have lower differentiation capacity than bone marrow derived cells.
- Growth factor cocktail differentiation methods yielded expression of β-tubulin-III but no other neural markers.
- Co-culture with astrocytes is the most promising differentiation method due to long-term cytotoxicity with the RA method and poor protein expression with growth factor method.

5. Month 12: Final report

*Unanticipated Challenges:*

The interference of chitosan with most commercially available cell viability assays presented us with an unexpected challenge and delayed completion of milestone number 2. We were able to optimize the commercially available CyQUANT assay for this purpose. In addition, electrospinning chitosan solutions into continuous fibers with no globules has proven very difficult. We have attempted to vary solution concentration, chitosan molecular weight, applied voltage and separation distance to optimize results. We have also tried to stabilize the chitosan solutions with PEO but have not been able to obtain continuous fibers. We did achieve a composite mat with collagen fibers and some chitosan fibers as well as globules but were not able to test for cell response to these matrices.

## ***Detailed Progress Report***

## *A. Stem Cell Efforts*

The stem cell effort seeks to (1) examine various conversion protocols in order to determine the most effective method of differentiating stem cells into neurons, (2) optimize techniques that will allow characterization of the differentiation process, and (3) determine whether collagen scaffolds support stem cells attachment and neuronal differentiation. We focused our initial efforts on Bone Marrow Mesenchymal Stem Cells (subsequently referred to in this report as MSCs).

Three different differentiation protocols have been examined in this effort to date and will be summarized in this report:

(1) Retinoic acid treatment (Cho et al., 2005), described in Task 1
(2) Preincubation with Epidermal Growth Factor (EGF) and basic Fibroblast Growth Factor (bFGF) followed by addition of Retinoic Acid (RA), Brain Derived Neurotrophic Factor (BDNF), Nerve Growth Factor (NGF) and Neurotrophin-3 (NT-3) (Hermann et al., 2004), described in Task 2
(3) Co-culture with astrocytes (Bossolasco et al., 2005), described in Task 3

In addition, we have investigated the effect of collagen 2D and 3D substrates on MSC protein expression (Task 1).

**Task 1: Differentiate Adult Stem Cells into Neurons. Each of three different adult stem cell types (ADSCs, MSCs and amniotic-derived stem cells) will be investigated for the ability to differentiate into neurons in monolayer. (10 months)**

In order to compare differentiation protocols and characterize the differentiation process, the expression of certain neuron-specific proteins has been examined. It has been shown that untreated MSCs express the neural progenitor marker, nestin, which indicates their potential to differentiate into neurons. We verified nestin expression in our MSC cell source (data not shown.) Studies have widely demonstrated that MSCs can be induced to express the neuron-specific proteins β-tubulin III, NeuN and GFAP at early stages of differentiation (within 2 weeks) while it is possible to find late stage markers like MAP2ab in the terminal stages of differentiation, depending on the method used (Bossolasco et al., 2005; Cho et al., 2005; Hermann et al., 2004; Sanchez-Ramos, 2000; Wislet-Gendebien, 2005.) These common neuronal markers are summarized in Table 1.

**Table 1: Neuronal Markers Function and Expression Time Frame.**

| Marker | Function | Type |
|---|---|---|
| Nestin | Neural intermediary filament type IV. It is an important cytoskeletal component of neuronal axons and seems to have an influence over axonal diameter. | neural progenitor marker (early) |
| βtubulin III | microtubule subunits in neurons | Immature neuronal phenotype |
| NeuN | Soluble nuclear protein that binds to neural DNA. It is exclusive to tissue in the nervous system, and is only expressed by the neurons in the nervous system. | Intermediate neuronal phenotype |
| MAP2ab | Neuronal phosphoprotein that promotes net microtubule growth and actin cross-linking and bundling in vitro. | Late neuronal phenotype |
| GFAP | Type III protein of the intermediate filament exclusively found in astrocytic cells of the central nervous system | Glial phenotype |
| synaptophysin | A calcium-binding glycoprotein that is present in the pre-synaptic vesicles of neurons and in the neurosecretory granules of neuroendocrine cells. | Synaptic transmission |

*Neural differentiation of MSCs via retinoic acid (RA) treatment in monolayer and on a three-dimensional collagen scaffold.* MSCs cultured in monolayer and on a three-dimensional collagen sponge were treated with retinoic acid (RA) for up to 14 days and analyzed by phase-contrast microscopy and immunofluorescence in order to assess the development of any neural morphology or expression of neuron-specific proteins in response to RA exposure. Morphological changes could be detected after 3 days of RA treatment as they lost their symmetrical shape, and exhibited phase-bright, spherical cell bodies with long extensions by day 6 (Figure 1A). After 14 days of treatment, MSCs demonstrated neuron-like unipolar and bipolar morphologies (Figure 1B).

A


B

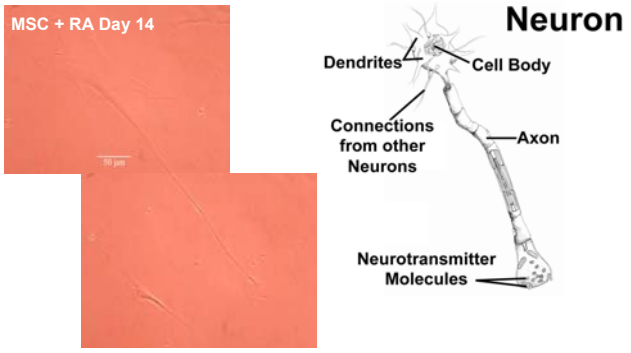



**Figure 1. Phase-contrast microscope images showing the morphological change of RA-treated MSCs over a 14 day-period. (A): MSCs before RA-treatment (left) exhibit symmetrical morphology. MSCs after RA-treatment on days 3 and 6 have increasingly spherical and refractile cell bodies (middle and right). Arrows point to cell body and extension. (B): RA-treated MSCs have cell bodies with dendritic and axon-like processes. Scale bar: 50 µm.**

After 8, 12, and 14 days of exposure to RA, MSCs were tagged by fluorescent antibodies specific to β-tubulin III, GFAP, MAP2, and synaptophysin and examined under a confocal microscope. After 8 days of exposure to RA, MAP2 and synaptophysin staining was detected in MSCs grown both in monolayer and on a collagen matrix (Figure 2). Furthermore, staining for these proteins revealed the neuron-like morphology of treated MSCs, characterized by long processes and asymmetrical cell shape by day 12. However, after 14 days of RA treatment, scattered nuclear staining was observed in monolayer samples. An MTT assay was performed to assess cell viability of RA-treated MSCs over 20-day period. RA-treated MSCs exhibited a decrease number of viable cells after 12 days (data not shown). The number of viable, untreated MSCs also declined after 12 days, but after a peak in number of viable cells that was significantly greater than that observed in treated MSCs.

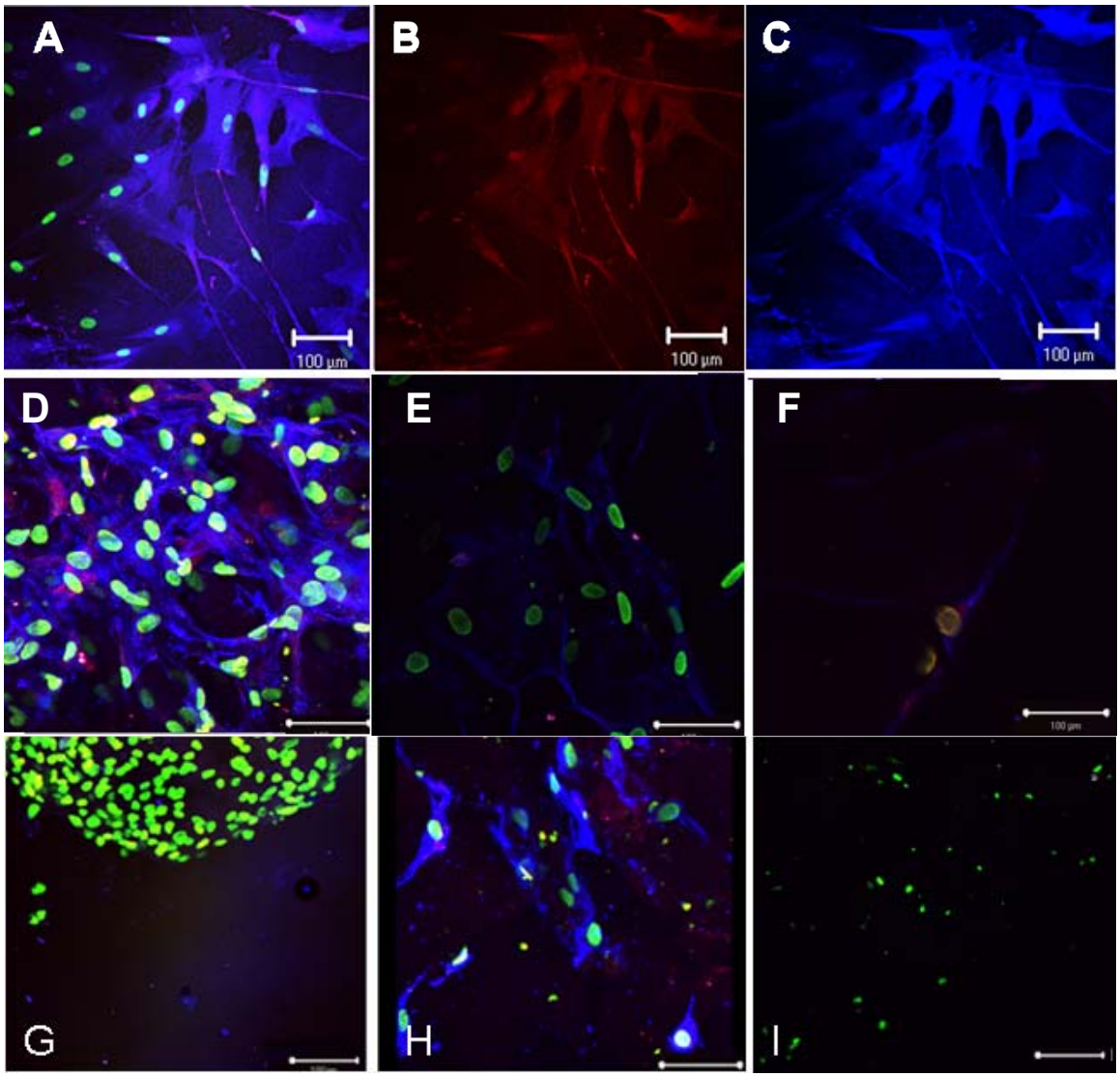



**Figure 2. RA-treated MSCs cultured in monolayer and on a collagen scaffold for up to 12 days, labeled for neuron- and astrocyte-specific proteins and detected by confocal microscopy. Cell nuclei are labeled with a Sytox green stain. MAP2 and synaptophysin expression co-located (purple) in RA-treated MSCs grown in monolayer for 8 days (A). Single-color scanning showed bright synaptophysin staining (red) (B) and MAP2 staining (blue) (C). RA-treated MSCs on a three-dimensional collagen scaffold also stained positive for MAP2 after 8 and 12 days (D-E) and stained dimly for synaptophysin after 12 days (F). After 8 days, MSC only control samples in monolayer were negative for MAP2 and synaptophysin (G), but stained brightly for MAP2 when cultured on collagen (H). After 12 days, MSCs on collagen were negative for MAP2 and synaptophysin (I). Sample size, n=2. Scale bar: 100 µm.**

*Effect of collagen on neural differentiation of MSCs.* MSCs alone were cultured on 3D alginate scaffolds, 2D collagen films, and 3D collagen matrices in order to determine whether collagen influences the neural differentiation of MSCs by providing biological or structural support. While the alginate scaffolds remained intact throughout the culture period, they disintegrated during immunostaining, preventing analysis of protein

expression. After 4 weeks in culture, MSCs on a 2D collagen film were negative for all protein markers, while MSCs on a 3D collagen matrix stained brightly for β-tubulin III (Figure 3). After 5 weeks in culture, MSCs on a collagen film and collagen matrix exhibited dim staining for β-tubulin III. All samples were negative for MAP2, synaptophysin and GFAP. Additionally, MSCs with and without astrocytes were cultured on a 2D collagen film for 2 and 3 weeks. After 2 weeks, neither condition gave rise to neuron-specific or astrocyte-specific expression. After 3 weeks, co-cultured MSCs expressed β-tubulin III while MSCs alone were negative. All samples were negative for MAP2 and synaptophysin at these times.

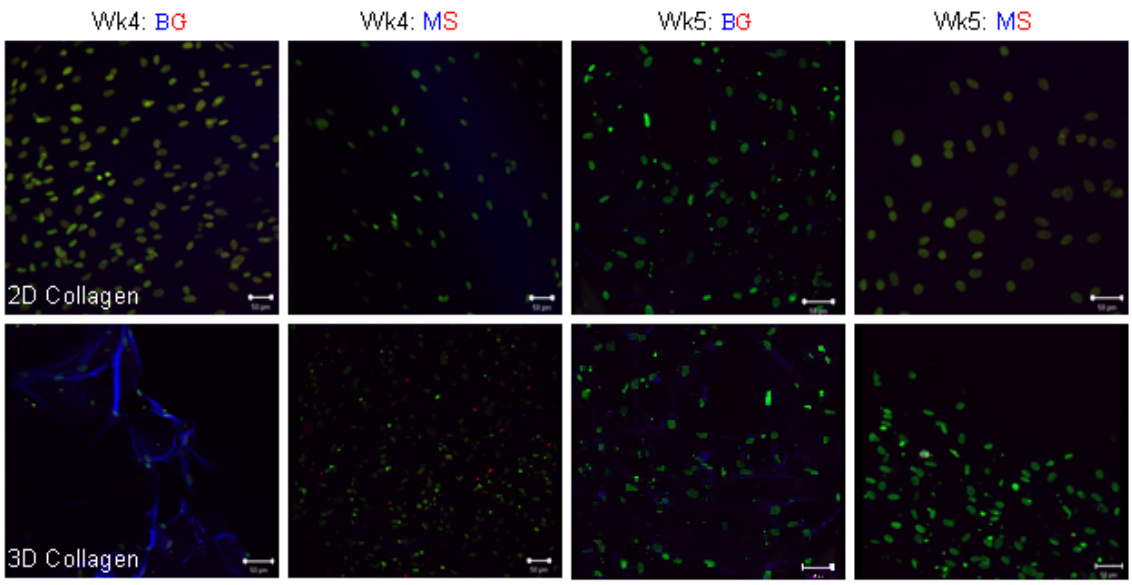



**Figure 3. Neuron-specific protein expression in MSCs cultured on a 2D collagen film and 3D collagen matrix after 4 and 5 weeks. Cells were either double-labeled for β-tubulin III (blue) and GFAP (red) or MAP2 (blue) and synaptophysin (red). MSCs cultured alone on a 3D collagen matrix express β-tubulin III at high levels after 4 weeks, and lower levels after 5 weeks. No β-tubulin III expression is detected in MSCs cultured alone on a 2D collagen film after 4 weeks, although dim staining is detected after 5 weeks. MAP2 is not detected in any samples in which MSCs were cultured alone. All samples were negative for GFAP or synaptophysin. BG: β-tubulin III, MS: MAP2/synaptophysin. Scale bar: 50 μm.**

## Task 2: Conduct culture studies on stem cells with neurotrophic factors added exogenously to the media (5 months)

We have also evaluated neural differentiation by addition of growth factor cocktails. Our initial attempt involved evaluating the timing of growth factor introduction to Mesenchymal Stem Cells (MSCs). In this study we compared pre-incubation with Epidermal Growth Factor (EGF) and basic Fibroblast Growth Factor (bFGF) followed by addition of Retinoic Acid (RA), Brain Derived Neurotrophic Factor (BDNF), Nerve Growth Factor (NGF) and Neurotrophin-3 (NT-3) versus addition of everything mentioned above at the same time (see table 2 for the function of each growth factor.)

Samples after one, two and three weeks differentiation were evaluated with phase-contrast microscopy and immunofluorescence of neural and astrocyte-specific markers. The positive control cells for immunofluorescence are rat brain cortex neuronal cells (see Task 4) and the negative control are undifferentiated MSCs. In addition, we investigated the neural supplement B-27 as a potential inducer of neural differentiation (Brewer et al., 1993; Lee J et al., 2003).

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| Growth Factor | Function |
|---|---|
| EGF (Epidermal Growth Factor) | Maintains cell proliferation |
| bFGF (basic Fibroblast Growth Factor) | Supports self-renewal at high concentrations, plays essential role in long-term hESC maintenance; may synergize w/collagen to induce differentiation of all CNS lineages. |
| BDNF (Brain-Derived Neurotrophic Factor) | used for neuronal induction |
| NT-3 (NeuroTrophin 3) | Promotes survival and neurite outgrowth from a subset of neural crest and placode-derived neurons; regulates proliferation of cultured neural progenitors in serum-free medium. |
| NGF (Nerve Growth Factor) | Induces differentiation and survival of neurons |

**Table 2. G**rowth factors used for neural induction

*Timing: pre-incubation with bFGF & EGF vs. addition of cocktail of growth factors.*

In this study we compared pre-incubation with Epidermal Growth Factor (EGF) and basic Fibroblast Growth Factor (bFGF) followed by addition of Retinoic Acid (RA), Brain Derived Neurotrophic Factor (BDNF), Nerve Growth Factor (NGF) and Neurotrophin-3 (NT-3) versus addition of everything mentioned above at the same time. After 3 weeks in culture, all samples were negative for neural markers MAP2 and synaptophysin (Figure 4.) Interestingly, control samples of MSCs in rat neuron media formed neurospheres after 3 weeks in culture.

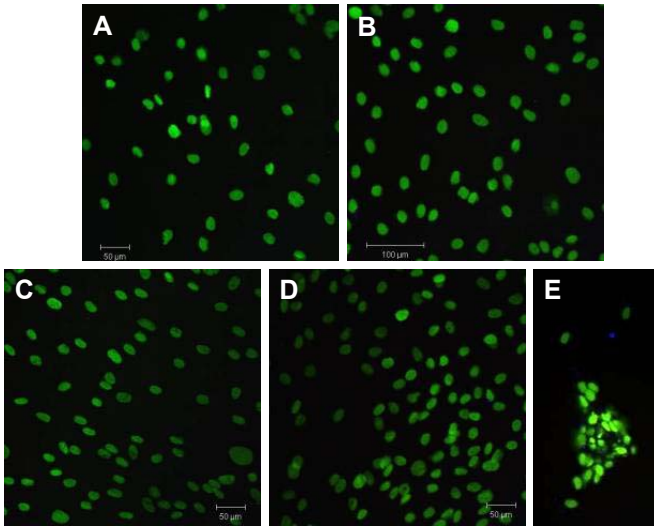

**Figure 4: Immunofluorescence staining of A) MSC only, B) MSC+RA, C) Pre-incubation with EGF and bFGF, D) Growth factor cocktail, and E) MSC in rat neuron media. All samples were negative for Synaptophysin (red) and MAP2 (blue) after 3 weeks in 2D culture.**

*Addition of BDNF only vs. growth factor cocktail during differentiation*

In this experiment, we compared differentiation with BDNF vs. growth factor cocktail (BDNF, bFGF, EGF, NGF, NT-3) following neurospheres formation in rat neuron media. Retinoic acid was added for 3 days during neurospheres formation and 4 days during initial differentiation in BDNF or growth factor cocktail. After 3 weeks in culture, both groups were positive for β tubulin III (TUJ-1) but negative for neural markers MAP2 and synaptophysin (Figure 5.)

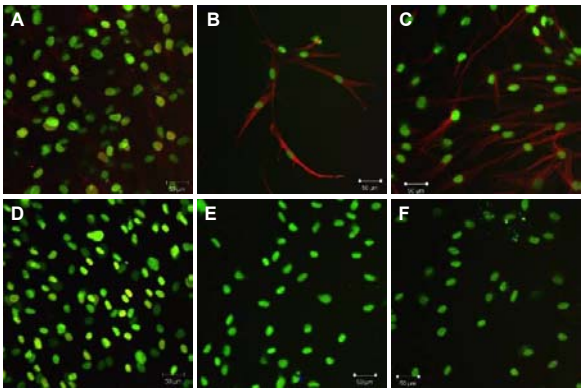


**Figure 5. Immunofluorescence staining of doublestaining β tubulin III (red)-MAP2 (blue) top panels and doublestaining Synaptophysin (red)-MAP2 (blue) bottom panels of A, D) MSC only, B, E) BDNF in differentiation media, C, F) growth factor cocktails in differentiation media after 3 weeks culture. Samples were positive for β tubulin III whether BDNF only or growth factor cocktails were added in differentiation media but they were negative for MAP2 and synaptophysin.**

*Neurobasal Media + B-27 supplement*

Based on the literature, we wanted to try Neurobasal media plus the supplement B27. For efficient neurosphere formation, we seeded both MSCs and ADSCs on non-tissue culture-treated plates in serum-free neurobasal media+B27+bFGF+EGF+BDNF. After 3 days, the neurospheres were transferred to laminin-coated plates and differentiated in neurobasal media+B27 without the growth factors. All samples were negative for β tubulin III, MAP2 & synaptophysin (Figure 6.)

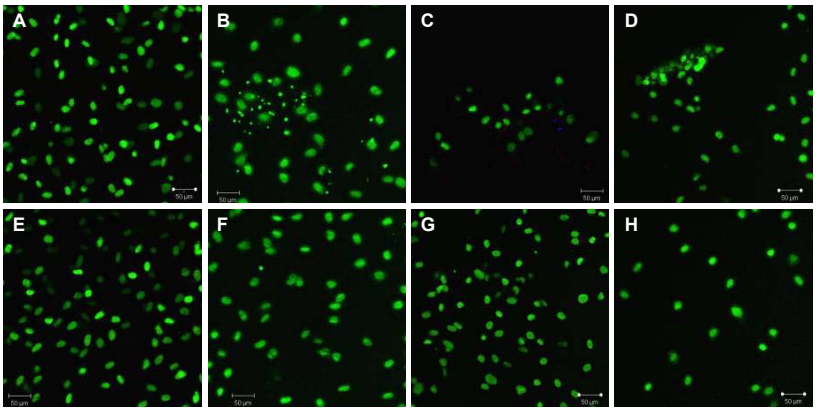


**Figure 6. Immunofluorescence staining of doublestaining β tubulin III (red)-MAP2 (blue) top panels and doublestaining Synaptophysin (red)-MAP2 (blue) bottom panels of A, E) undifferentiated MSC; B, F) differentiated MSC; C, G) undifferentiated ADSC; D,H) differentiated ADSC after 3 weeks culture. Samples were negative for β tubulin III, MAP2 and synaptophysin.**

**Task 3: Conduct culture studies on stem cells co-cultured with astrocytes and macrophages (5 months)**

*Neural differentiation of MSCs via astrocyte co-culture in monolayer and on a three-dimensional collagen sponge.* MSCs were co-cultured with astrocytes in non-contact conditions and immunostained for neuron-specific and astrocyte-specific markers after 4 weeks. MSCs co-cocultured with astrocytes for 4 weeks showed dim staining for β-tubulinIII in monolayer and bright staining on a 3D collagen matrix. MAP2 fluorescence was detected in the three-dimensional co-culture, but no staining was detected in monolayer samples at this time point (data not shown.)

*Effect of cell density on neural differentiation of MSCs by astrocyte co-culture method.* MSCs were plated, with and without astrocytes, at a low density of 2,500 cells/cm$^2$ and high density of 4,000 cells/cm$^2$ in monolayer and on a collagen film. For three-dimensional culture, MSCs were seeded on collagen scaffolds at 50,000 cells/cm$^2$ and 80,000 cells/cm$^2$. Co-cultured MSCs in monolayer and on a collagen film were negative for all neuron- and astrocyte-specific protein markers. There was no significant difference in β-tubulin III expression between co-cultured MSCs plated at low and high density on a collagen sponge at weeks 2, 3, and 4 (Figure 7). Both sets of MSCs stained brightly for β-tubulin III at week 2 and then dimly at weeks 3 and 4. However, MSCs cultured alone at high density exhibited a peak in β-tubulin III expression earlier than MSCs at a lower density. At high density, MSCs exhibited bright staining at week 2, dim staining at week 3 and no staining at week 4. In contrast, MSCs cultured alone at low density exhibited a peak in β-tubulin III expression at week 4. All samples were negative for GFAP expression. MAP2 staining was detected in co-cultured MSCs at high density on weeks 3, 4, and 5, and only at week 5 in MSCs at low density (data not shown.) MSCs cultured alone were negative for MAP2 at all time points. All samples were negative for synaptophysin at each time point.

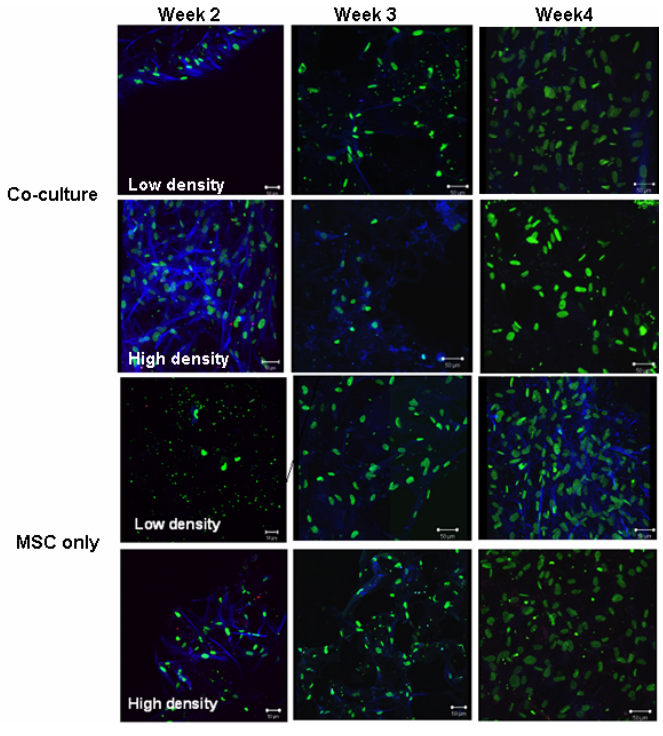


**Figure 7. Effect of MSC density on β-tubulin III expression in MSCs cultured with and without astrocytes on a three-dimensional collagen at weeks 2, 3, and 4. Co-cultured MSCs at both densities exhibited bright staining for β-tubulin III (blue) at week 2, which decreased at weeks 3 and 4. MSCs cultured alone at a high density stained brightly for β-tubulin III at week 2, while β-tubulin III expression in MSCs at a lower density did not peak until week 4. All samples were negative for GFAP (red). Scale bar: 50 μm.**

**Task 4: Develop Assays for Neuronal Cell Surface Markers. Characterize the cultured neurons using investigative techniques such as confocal microscopy, ELISA, and Western blotting. (2 months)**

We have thoroughly characterized all cellular markers for immunofluoresence, including positive controls for each marker (Figure 8). In order to more quantitatively determine neuronal protein expression in differentiated cells, we optimized Western Blot protocols for β-tubulin III, MAP2, Synaptophysin and GFAP. Rat brain cortex neuronal cells were used as positive controls for neuron-specific proteins as well as glial specific GFAP for determination of optimal total protein concentrations and antibodies concentrations for the assay. We have determined optimal protein loading amounts and antibody concentrations for the detection of all four neural specific proteins using the Western Blot method (Figure 9.)

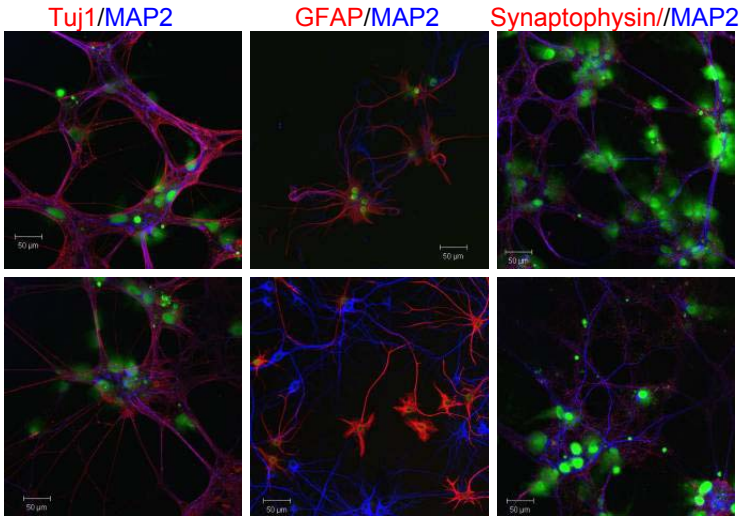


**Figure 8. Rat brain cortex neuronal cells stained for neuron-specific proteins β-tubulin III (Tuj1), MAP2, synaptophysin and astrocyte marker GFAP. Cells are double-labeled for β -tubulin III (red) and MAP2 (blue) (left), GFAP (red) and MAP2 (middle), and synaptophysin (red) and MAP2 (right). Nuclei are stained with Sytox green.**

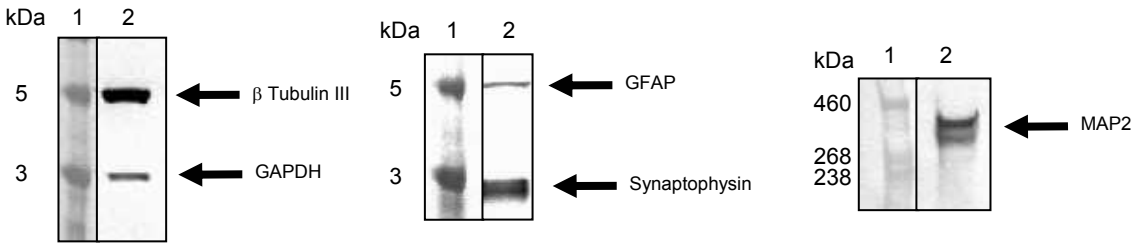


**Figure 9. Western blot analysis of neural and astrocytic markers in rat brain cortex neuronal cells (positive control cells). A) Beta tubulin III (intermediate neuron) and GAPDH (housekeeping gene) bands; B) GFAP (astrocytic) and Synaptophysin (mature neurons) bands; C) MAP2 (mature neurons) bands. In fig. A-C lane 1 are protein ladders, lane 2 are rat neurons lysates.**

## ***B. Cell Scaffold Efforts***

**Task 1: Develop composite hydrogel. Optimize the collagen:chitosan ratio for neuronal cell culture, evaluate cross-linking methods, and design gel fabrication procedures. (4 months)**

Significant effort has been directed to developing hydrogel composites of collagen and chitosan in order to optimize the conditions conducive to cell growth and differentiation. While collagen provides a stable platform for cell growth, chitosan-based materials may contribute to mitigation of inflammation and stimulate growth. Various ratios of these compositions, including a chitosan-guanidine derivative, are currently being assessed. We have evaluated two types of scaffolds: one from a mixture of chitosan with collagen and one from chitosan-guanadine, which show great promise as cell substrates.

*Chitosan-Guanadine Scaffold Development.* To synthesize chitosan - guanidine scaffolds, 1.0g powdered Primex chitosan (85% deacetylation) is added to 20mL of Millipore water and 80mL of 1% acetic acid. This mixture is left on a shaker overnight. After shaking, 5mL of solution is poured into each well of a 12-well tissue culture plate. The plates are freeze-dried for three to four days to prevent the formation of ice crystals and preserve the structure of the sample. To prepare a chitosan gel from a scaffold, the sample is first massed then swollen with 1.0ml of cell media for every 0.14 g of scaffold. A probe is used to compress the sample to fully wet the structure. After swelling, the chitosan ball is placed in an incubator at 37°C for two hours, after which, 5.0mL of media is added to cover the gel which is returned to the incubator for a 24 hour period. The gel is then washed by removing the media around the gel and replacing it with 5.0mL of fresh media. The gel is incubated for another two hours, washed again, and then incubated for an additional 24 hours. The washing and incubating cycle raises the pH from 5.5 to 7 in order to provide an appropriate environment for cell growth. The sample is freeze dried a final time and rehydrated prior to cell seeding (Figure 10).

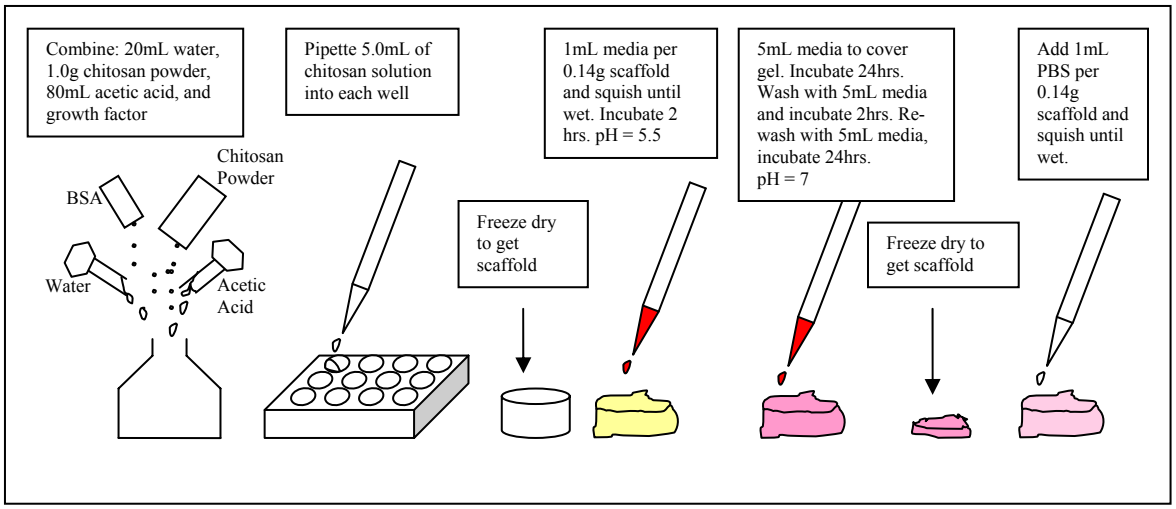


**Figure 10: Chitosan –guanidine sample preparation.**

*Chitosan-Collagen Composite Scaffold Development.*
Chitosan-Collagen composite scaffolds were prepared using a lyophilization process. Avitene ultrafoam collagen sponges (#1050020) were purchased from Davol Inc. and cut into small pieces to fit plate wells (2cm for 12-well plate, 1cm for 24-well plate). Chitosan-guanidine was prepared using powdered Primex chitosan (85% deacetylation), as described above. 5 mL of chitosan-guanidine solution was then poured on top of the Avitene collagen sponges into each well for a 12 well plate (2.5 mL for 24 well plate). Samples were allowed to sit for 1 hour before lyophilization to obtain a pH 5.5 scaffold. The resulting scaffold was then neutralized to pH 7 according to the methods described above.

*Cell Viability Testing.* Preliminary cell viability testing on our developed matrices showed promising results, which were reported in our initial report. Further experiments resulted in a surprisingly high background signal from our chitosan and chitosan-guanadine scaffolds, leading us to suspect that chitosan may interfere with the MTT assay by contributing to the reduction of the colorometric agent involved. We conducted an experiment that verified this hypothesis. As a result of the interference of chitosan with the MTT assay, we began optimizing the protocol for another cell viability assay, the CyQUANT® NF Cell Proliferation Assay Kit (Invitrogen.) The CyQUANT® NF assay is based on measurement of cellular DNA content in a sample via fluorescent dye binding. This assay is advertised to be more accurate because cellular DNA content is highly regulated and therefore more closely proportional to cell number. This assay does not require the use of radioisotopes, enzymes, or antibodies and is not dependent on physiological activities that may exhibit cell number–independent variability. We have created a standard curve using our cells and the CyQUANT assay which is linear (Figure 11). Initial attempts to assay cell viability on three-dimensional scaffolds were unsuccessful due to diffusion limitations in thick three-dimensional samples. This problem was not chitosan-specific as the collagen control samples also did not produce a significant signal. Modification of the sample preparation protocol to include a

proteinase K digestion step led to more promising results (Figure 12). Proteinase K acts to break the cell attachment to the scaffold, stabilize the DNA and increase Cyquant fluorescence signal. As shown in Figure 12, the Cyquant assay on chitosan samples with proteinase K digestion led to increased signal and a linear standard curve.

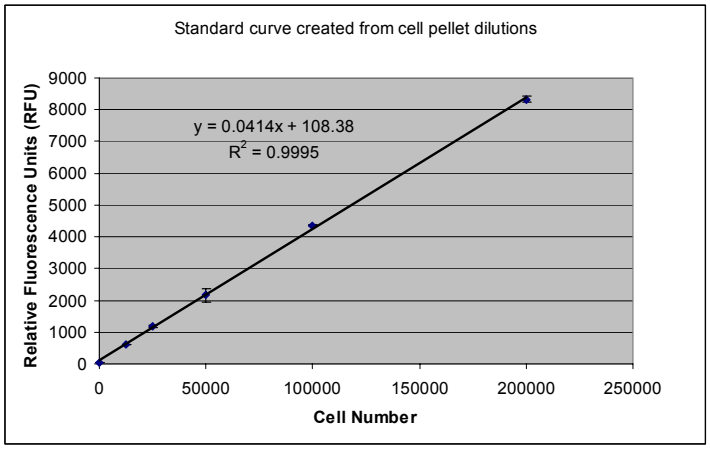


**Figure 11: Standard Curve for CyQuant Assay**

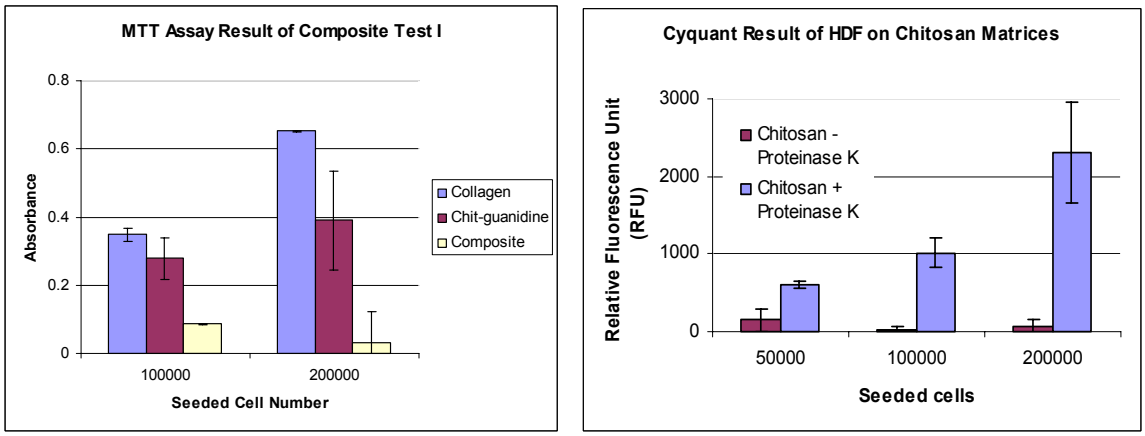


**Figure 12. Comparison of MTT and Cyquant assay results on collagen, chitosan-guanidine and composite samples. The MTT assay (left) was not reproducible on either chitosan scaffold and yielded nonlinear results. The Cyquant assay with the addition of proteinase K digestion led to an increased signal which is linear ($R^2$ = 0.99) in chitosan samples.**

Cell viability of MSCs grown on collagen, chitosan-guanidine and composite chitosan-collagen scaffolds were also evaluated using confocal microscopy with a nuclear stain and scanning electron microscopy. In confocal microscopy images, attached cell number appears approximately equivalent in day 1 and day 3 samples, however cell nuclei appeared more aggregated on chitosan-based samples (Figure 13, top two rows.) By day 7 in culture, collagen samples exhibit dense cell attachment and nuclei are evenly distributed, while chitosan-based samples exhibit fewer attached cells and more aggregated nuclei (Figure 13, third row). By day 14 in culture, there are marked

differences between collagen and chitosan-based scaffolds, indicating that long-term cell survival may be an issue on these scaffolds (Figure 13, bottom row). SEM results corroborate the confocal images (Figure 14.) Both confocal and SEM results show that cells initially attached to all scaffolds, but significant long-term attachment of MSCs was not observed on either chitosan-guanidine or on the composite scaffold. This longer term cell viability issue may be related to the use of commercial grade chitosan sources in the preparation of the chitosan-based scaffolds. These commercial grade chitosans have a high endotoxin burden, which may be contributing to cell death after a week in culture. Future studies will investigate the use of low endotoxin chitosan sources for composite development.

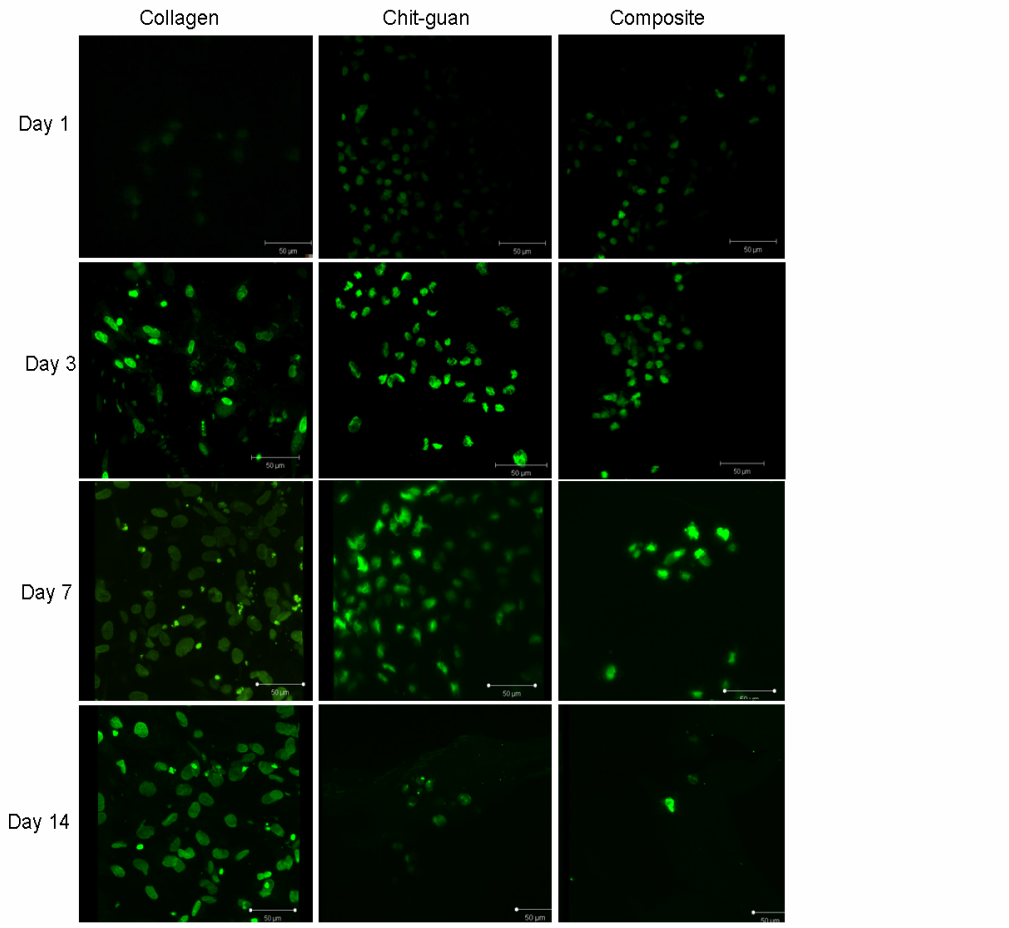


**Figure 13. Confocal microscopy of MSCs grown on collagen, chitosan-guanidine and chitosan-collagen composite scaffolds. Day 1 and Day 3 images show equivalent cell attachment on all 3 scaffolds. Day 7 chitosan-guanidine samples have more cell aggregation than collagen samples and chitosan-collagen composites have fewer cells. By Day 14, both chitosan-based scaffolds have markedly fewer cells than collagen scaffolds.**

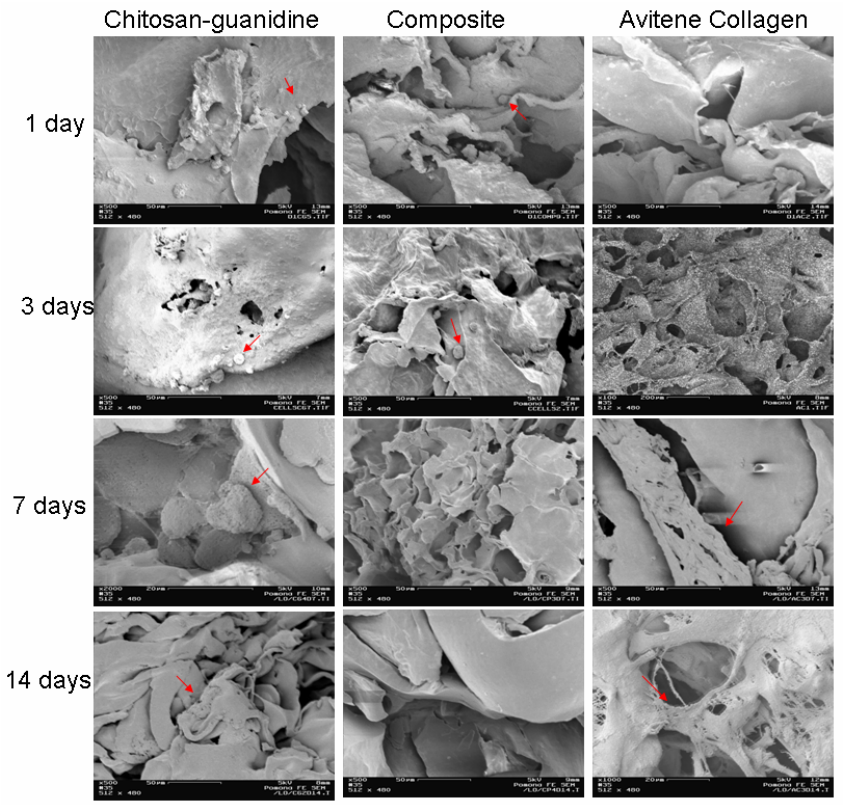


**Figure 14. SEM images of MSCs grown on collagen, chitosan-guanidine and chitosan-collagen composite scaffolds. Day 1 and day 3 samples show cells scattered across the surface, usually along the ridges of the scaffold with spherical morphology. By day 7, collagen samples had many attached cells, while chitosan-based samples had fewer cells. On day 14, collagen samples show spread cells and evidence of ECM production, while fewer cells are observed on chitosan-based samples.**

**Task 2: Characterize the gel properties. Evaluate gel material properties such as liquid to gel transition temperature, fiber and pore sizes, mechanical strength, resistance to shear forces, and degradation rate. (2 months)**

*Structure and pore sizes.* Avitene Ultrafoam Collagen Hemostat was obtained from Davol (Cranston, RI) and provided a control scaffold for our studies. Scanning electron microscopy images showed the surface structure of the collagen, chitosan-guanidine, and chitosan-collagen composite scaffolds prior to cell seeding, before and after hydration of the samples (Figure 15.)

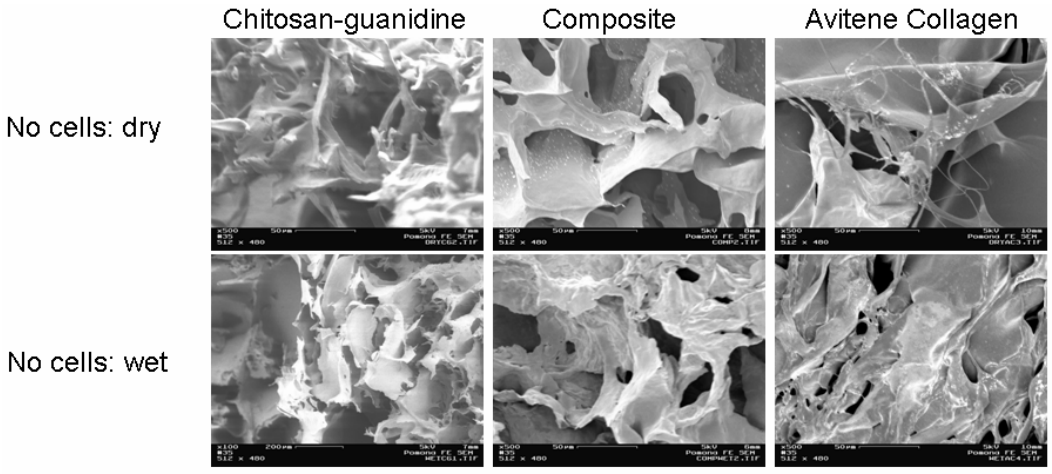


**Figure 15: Scanning Electron Microscopy (SEM) images of scaffolds before cell seeding. Top row: dry scaffolds. Bottom row: scaffolds swollen in PBS prior to SEM preparation protocols to show differences in pore structure after hydration.**

*Diffusion of Proteins*. One of the goals of this project is to be able to characterize the release kinetics of neural-specific growth factors from matrices we have created. This will help to inform the design of an appropriate growth-factor laden matrix for the cell delivery system. We have done some preliminary analysis of protein diffusion through collagen and chitosan scaffolds to establish appropriate methods for these studies. Preliminary studies were done with Avitene collagen sponges and bovine serum albumin (BSA). BSA was chosen because its concentration can be quickly and easily measured using the Bio-Rad Protein Assay. Collagen sponge samples were prepared in two different ways. Generally, sponges were cut to fit the size of the culture plates, swollen in PBS and freeze dried. In one type of sample (freeze-dried BSA), BSA was added to the PBS solution and freeze-dried into the sample. In the second type of sample (swollen BSA), BSA was swollen into the sample after freeze drying and immediately prior to diffusion tests. Methods for measuring diffusion kinetics of growth factors in the gels in a diffusion cell apparatus as well as in a free cell configuration have been determined (Figure 16.) In both methods, samples are analyzed for BSA concentration. The standard curve for the BSA assay used to determine concentrations from our samples is shown in Figure 17. Once concentrations of NGF are determined, we can calculate diffusivity of our hydrogels. The experimental setup can be described as transient diffusion out of a film with infinite sink upstream and downstream and Fick's 2nd Law of Diffusion can be used:

$$\frac{\partial c_1}{\partial t} = D\frac{\partial^2 c_1}{\partial z^2} \qquad (1)$$

Where $c_1$ is the concentration of species 1 at the edge of the film, $D$ is the diffusivity, and $z$ is the film thickness. Equation 1 assumes that diffusion is one-dimensional and that there are no reactions in the film. It also assumes that the solubility is the same upstream and downstream, which is reasonable since the same solution will be placed on either side of the film. Lastly, Equation 1 assumes that the diffusivity is constant across the film. Although the hydrogels are non-homogeneous, an effective diffusivity for the bulk

media can be used so that the constant diffusivity assumption holds true (Crank, 1975.) To solve, one initial condition and two boundary conditions are needed. The initial condition is that the concentration is uniform across the thickness of the film and equal to $c_{10}$ (the concentration of growth factor initially swollen into the hydrogel). The boundary conditions are that there is infinite sink upstream and downstream for all time greater than zero (Crank, 1975.) From Fick's 2nd Law and the initial and boundary conditions, the following full solution for short times can be found:

$$\frac{M_t}{M_\infty} = 2\left[\frac{Dt}{\delta}\right]^{\frac{1}{2}}\left[\frac{1}{\sqrt{\pi}} + 2\sum_{n=1}^{\infty}(-1)^n \, ierfc\left(\frac{n\delta}{\sqrt{Dt}}\right)\right] \tag{2}$$

where $M_t$ is the mass of chemical released at time $t$ and $M_\infty$ is the mass of chemical released at time infinity. Equation 2 can be approximated as:

$$\frac{M_t}{M_\infty} \cong 2\left[\frac{Dt}{\pi\delta^2}\right]^{\frac{1}{2}} \tag{3}$$

The masses are determined experimentally so that this equation can be used to determine the diffusivity (Crank, 1975.) For a small changes in volume during the sampling process the volumes at $t = 0$ and $t = \infty$ are approximately equal. As such the equation reduces to:

$$\frac{c_t}{c_\infty} \cong 2\left[\frac{Dt}{\pi\delta^2}\right]^{\frac{1}{2}} \tag{4}$$

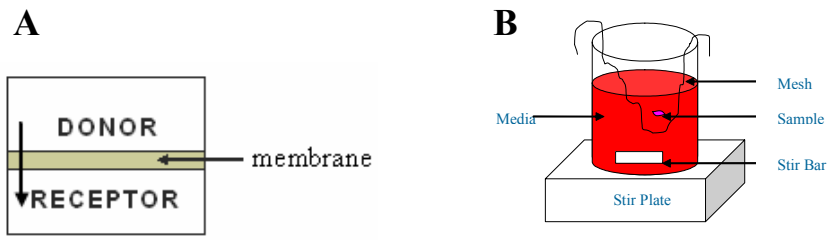


**Figure 16: A) Diffusion Cell experimental setup. The cell on the Hanson Microette Plus Vertical Diffusion Cell System consists of two main chambers separated by a membrane (the hydrogel in our case). The chemical diffuses out of the membrane and into the cell chambers. Saturated solution in the top chamber prevents flow into the donor compartment. The chemical is then automatically sampled from the receptor chamber. B) Dish test experimental setup. This setup analyzes the direct flow of proteins out of the hydrogels when immersed in an approximately infinite medium. The test setup involves 800 mL of solvent (PBS or cell media) in a beaker, and a mesh platform which is used to support the hydrogel at about half the depth of the surrounding fluid. The contents of the beaker are stirred at 400 rpm for the duration of the test to prevent saturation directly below the hydrogel. A sample of 200µL is collected manually at each sampling interval.**

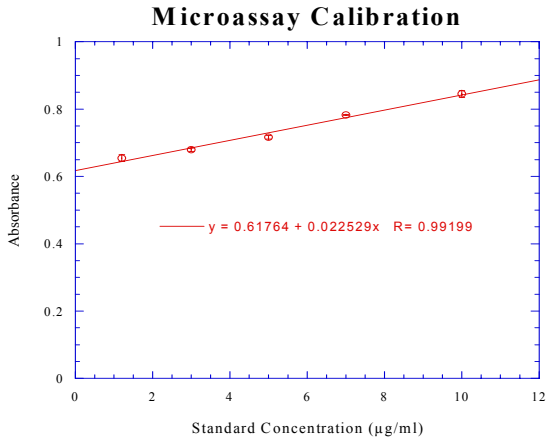


**Figure 17: BSA Microassay Calibration. Absorbances measured at standard concentrations with PBS as solvent. $R^2 = 0.9840$.**

We have calculated the diffusivity of BSA from collagen sponges using the dish test method (Figure 16B). The advantage of the dish test over the diffusion cells test is that samples can be taken much more quickly. The test shown in Figure 18A had a six minute run time with a ten second sampling interval. In this test, BSA was added after freeze drying (swollen BSA). There is a clear region with increasing concentration over the 6 minute run time. Another dish test was performed BSA added before the freeze drying step (freeze dried BSA) (Figure 18B.) Concentrations for these figures were obtained using the calibration shown in Figure 17. Using Equation 4, the diffusivity was calculated from the data shown in Figure 18. Figure 19 shows the fit to Equation 4. From the fit, a value for diffusivity and a value for error were determined. BSA diffuses out of collagen hydrogels much more quickly when it is simply swollen into the scaffold than when it is freeze dried into the structure. The diffusivity for the collagen sample with BSA freeze dried into the sample was significantly less than the diffusivity for the hydrogel with BSA swollen into the scaffold immediately before diffusion testing. These preliminary diffusion studies indicate half-lifes of diffusion are observed to be hours rather than days. Non-covalently bound materials may thus be possible for short-term stimulation/control of cell activity while covalently bound factors may be utilized for longer term stability or adhesion.

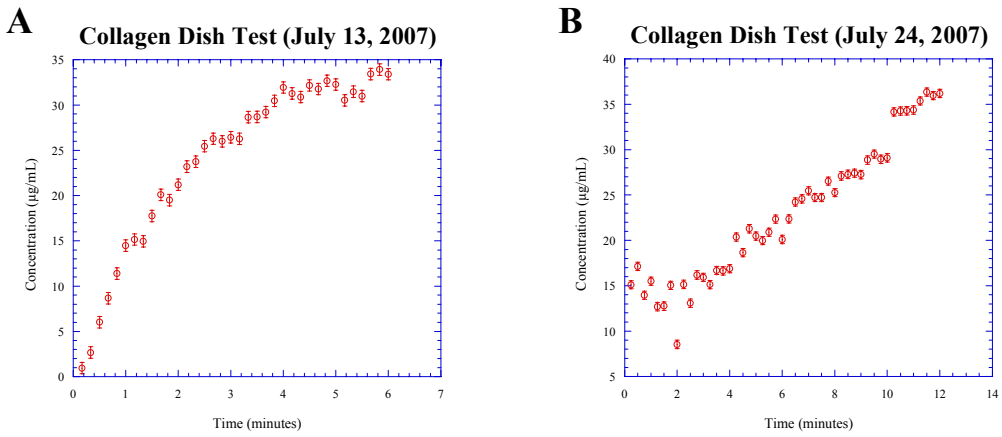


**Figure 18: Collagen Dish Tests. A. Swollen BSA. The change in absorbance of BSA as a function of time in the dish test performed in PBS with a sampling interval of 10 seconds. The collagen was swollen with BSA after freeze drying (swollen BSA) and the total run time was six minutes. B. Freeze dried BSA. The sampling interval was 15 seconds for a total run time of 12 minutes. The collagen was swollen with BSA before freeze drying (freeze dried BSA).**

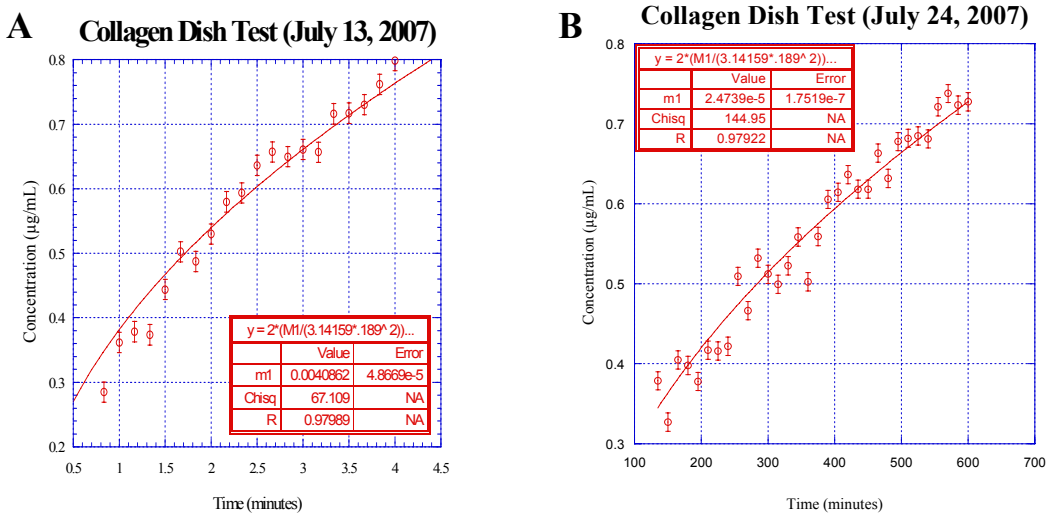


**Figure 19. Collagen Dish Test Diffusivity calculations. Using Equation 4 and fitting it to the data, the values for diffusivity are (A) $4.086 \times 10^{-3}$ and (B) $2.474 \times 10^{-5}$.**

**Task 3: Electrospin Chitosan-Collagen Composite Conduits. Electrospin chitosan alone, then modify these techniques to create small fibril diameter, aligned chitosan-collagen composite conduits. (4 months)**

Our effort to electrospin chitosan focused on electrospinning in non-volatile solvents, which is a difficult task. We have been working on electrospinning chitosan fibers and chitosan-collagen composite mats. We have created electrospun collagen nano-fibers (Figure 20), chitosan nano-fibers (Figure 21), and composite collagen and chitosan nano-fibers (Figure 22). We have attempted to vary solution concentration, chitosan molecular weight, applied voltage and separation distance to optimize results. In an attempt to electrospin more continuous chitosan fibers, we stabilized the solution by addition of PEO prior to electrospinning. Figure 23 shows that we were not able to produce fibers using this method.

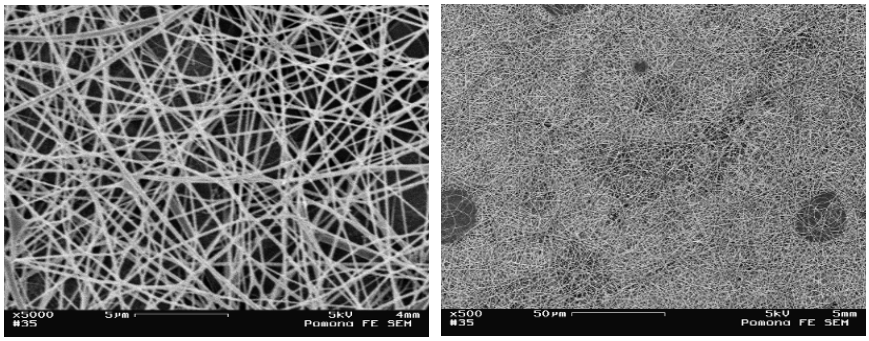


**Figure 18: Collagen nanofibers electrospun from a 6% solution of type I collagen in acetic acid.**

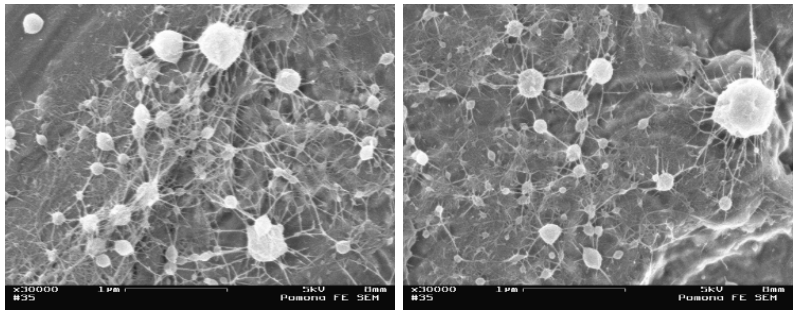


**Figure 19: Electrospun chitosan deposits forming short fibers as well as large globular particles, electrospun from a 1% solution of Chitosan in acetic acid and methanol.**

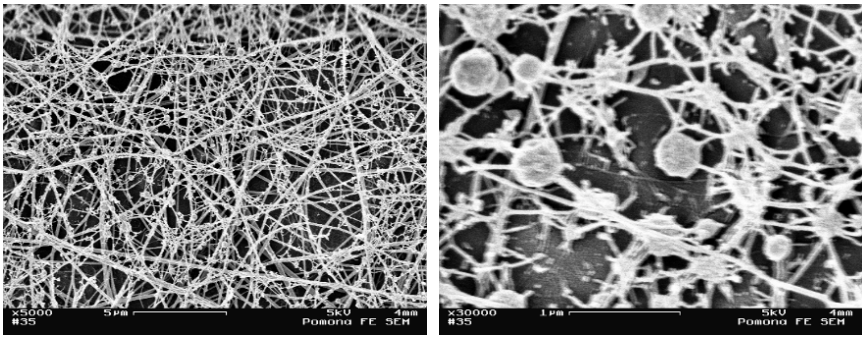


**Figure 20: Chitosan electrospun on top of collagen fibers formed from a 1% solution of chitosan and 6% solution of Type I collagen. Although chitosan did not form fibers, it can be seen that the chitosan globules have extended small fingers that attach to the larger collagen fibers.**

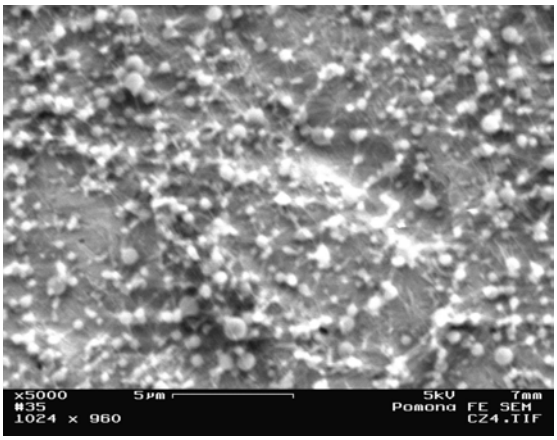


**Figure 23. 1% Chitosan-1% PEO solution electrospun in acetic acid. Continuous fibers are not observed.**

**Task 4: Characterize Conduit Properties. Evaluate conduit properties including fibril diameter and alignment, interaction between chitosan and collagen fibers, mechanical properties, and degradation rates. (2 months)**

From scanning electron microscopy images, we determine that the fibers produced in our electrospun chitosan matrices are approximately 30-60nm in diameter (Figure 21.) In addition, the fibers are short and extend from larger several hundred nanometer globular structures. In composite chitosan-collagen electrospun mats, these shorter fibers appear to extend and connect with the larger diameter collagen fibers. Electrospinning chitosan in non-volatile solvents presented a significant time-intensive challenge. As such, we were unable to evaluate these mats more extensively. In addition, gels were determined to be easier to manipulate and control in terms of overall structure and shape. Next steps for electrospun mats include crosslinking, evaluation of degradation, and cell viability testing.

**References**


Bossolasco P, L Cova, C Calzarossa, SG Rimoldi, C Borsotti, G Lambertenghi Deliliers, V Silani, D Soligo, E Polli. Neuro-glial differentiation of human bone marrow stem cells in vitro. *Experimental Neurology* 193: 312-325 (2005).

Brewer GJ, Torricelli JR, Evege EK, Price PJ. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J.Neurosci Res* 35(5): 567-76 (1993).

Cho K, KA Trzaska, SJ Greco, J McArdle, F Wang, J Ye, and P Rameshwar. Neurons derived from mesenchymal stem cells show synaptic transmission and can be induced to produce the neurotransmitter substance P by interleukin-1α. *Stem Cells.* 23: 383-391 (2005).

Crank, John. *The Mathematics of Diffusion 2nd ed*. Oxford, UK: Clarendon Press, 1975.

Hermann A, Gastl R, Liebau S. Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells. *Journal of Cell Science* 117: 4411-4422 (2004).

Lee J, Elkahloun A, Messina S. Cellular and Genetic Characterization of Human Adult Bone Marrow-Derived Neural Stem-Like Cells: A Potential Antiglioma Cellular Vector. *Cancer Research* 63:8877-8889 (2003).

Sanchez-Ramos, JR. Neural cells derived from adult bone marrow and umbilical cord blood. *Journal of Neuroscience Research*. 69: 880-893 (2002).

Wislet-Gendebien S, F Wautier, P leprince, B Rogister. Astrocytic and neuronal fate of mesenchymal stem cells expressing nestin. *Brain Research* 68:95-102 (2005).